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Phenotype-Associated *env* Gene Variation among Eight Related Human Immunodeficiency Virus Type 1 Clones: Evidence for In Vivo Recombination and Determinants of Cytotropism outside the V3 Domain

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The nucleotide sequences of the *env* genes of eight phenotypically heterogeneous human immunodeficiency virus type 1 (HIV-1) clones recovered from a single individual within a 3-week period were compared. In addition, the accessory gene sequences for four of these clones were obtained. Variation among most accessory genes was limited. In contrast, pronounced phenotype-associated sequence variation was observed in the *env* gene. At least three of these clones most likely resulted from genetic recombination events in vivo, indicating that this phenomenon may account for the emergence of proviruses with novel phenotypic properties. Within the *env* genes of the eight clones, four domains could be defined, the sequence of each of which clustered in two groups with high internal homology but 11 to 30% cluster variation. The extensive *env* gene variation among these eight clones could largely be explained by the unique manner in which the alleles of these four domains were combined in each clone. Experiments with chimeric proviruses demonstrated that the HIV-1 *env* gene determined the capacity to induce syncytia and tropism for T-cell lines. Amino acids previously shown to be involved in gp120-CD4 and gp120-gp41 interaction were completely conserved among these eight clones. The finding of identical V3 sequences in clones differing in tropism for primary monocytes and T-cell lines demonstrated the existence of determinants of tropism outside the *env* V3 region.

Human immunodeficiency virus type 1 (HIV-1) isolates possess distinct biological properties with respect to cytotropism, replication rate, and capacity to induce syncytia (4, 7, 30, 31). Previously, we have demonstrated that from stable asymptomatic individuals, only non-syncytium-inducing (NSI) HIV-1 variants which cannot be transmitted to the H9 or MT-2 T-cell lines can be recovered. In contrast, the detection of syncytium-inducing (SI) T-cell-line-tropic HIV-1 variants is strongly associated with rapid CD4 T-cell depletion and progression to AIDS (20, 31).

Recently, several studies have identified mechanisms and regions of the HIV-1 genome which control biological variation of HIV-1. We and others have shown that differences in cytotropism are determined at an early level of the virus replication cycle prior to provirus formation, which suggests involvement of the *env* gene (3, 13, 27). Studies with chimeric proviruses have formally identified the *env* gene as the major determinant responsible for differences in biological properties such as cellular tropism and cytopathogenicity (24, 28).

We previously described the generation of a panel of phenotypically distinct yet genetically highly homologous infectious molecular HIV-1 clones (13). These molecular clones were derived from HIV-1 isolates, mostly recovered by direct clonal isolation, from a single individual who developed AIDS within half a year after seroconversion. The clones were obtained from patient peripheral blood mononuclear cells collected at about the time of transition from an NSI to an SI HIV-1 phenotype. All eight clones were able to replicate in peripheral blood lymphocytes. The molecular and biological HIV-1 clones exhibited differences in SI capacity and in tropism for T-cell lines and monocyte-derived macrophages (13, 27). To genetically compare phenotypically heterogeneous HIV-1 variants and to analyze regions of the HIV-1 *env* gene previously identified as determinants of biological properties, we analyzed eight envelope sequences obtained from four infectious molecular HIV-1 clones (13) and from four biological HIV-1 clones through polymerase chain reaction amplification (1). In addition, we determined the sequences of the accessory genes of the four molecular clones. The biological phenotypes of the four molecular HIV-1 clones and the four biological clonal HIV-1 variants are shown in Table 1. Clones 320.2A 6, 320.2A 3, 320.2A 2.1, 320.2A 1.1, 320.2A 1.2, 320.2A 5, 320.2A 7, and 320.3 1 will be referred to as clones 6, 3, 2.1, 1.1, 1.2, 5, 7, and 1, respectively.

Although the SI HIV-1 clones differed in their abilities to replicate in the H9 and Sup T1 T-cell lines, all SI clones productively infected the MT-2 T-cell line, whereas NSI HIV-1 clones did not infect the MT-2 cell line (Table 1). This finding is in accordance with the strong correlation between the abilities of field isolates to induce syncytia and tropism for the MT-2 cell line (20). SI capacity also appeared to be required, although not sufficient, for H9 tropism (Table 1). In contrast, Sup T1 tropism was also observed for NSI clones, indicating that entry in Sup T1 cells is at least partially

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TABLE 1. Biological phenotypes of biological clonal HIV-1 variants and molecular HIV-1 clones derived from a single individual

Patient PBMC[a] sample	Clone no.[b]		PBL SI[c]	Transmission to:						MDM for replication[d]
	Biological	Molecular		MT-2		H9		Sup T1		
				Replication[e]	SI	Replication	SI	Replication	SI	
320.2A	6		−	−	−	−	−	+	−	NT
	3		−	−	−	−	−	−	−	NT
	2	1	−	−	−	−	−	++	−	+
	1	1	+	++	+	−	−	−	−	−
		2	(+)	++	+	−	−	−	−	−
	5		+	++	+	NT		+	+	NT
	7		+	++	+	+	+	+	+	NT
320.3		1	+	++	+	++	+	++	+	+

[a] PBMC, peripheral blood mononuclear cell.
[b] Biological clonal HIV-1 isolates were obtained from patient PBMC sample 320.2A, and molecular clones were derived from biological clonal HIV-1 isolates, except for clone 1.
[c] PBL, peripheral blood lymphocyte; −, no syncytia observed; (+), reduced SI capacity; +, SI.
[d] MDM, monocyte-derived macrophages; −, not transmissible; +, transmissible; NT, not tested.
[e] −, not transmissible; +, delayed growth kinetics; ++, readily transmissible; NT, not tested.

independent of the mechanisms involved in syncytium formation. Compared with clones 2.1 and 1, clones 5 and 7 exhibited delayed growth kinetics in the H9 and Sup T1 T-cell lines. In contrast to NSI Sup T1-tropic clone 2.1, NSI clone 6 replicated less efficiently in the Sup T1 cell line.

The alignments of the deduced amino acid sequences of the accessory genes are shown in Fig. 1. Except for the *vpu* gene of clone 1, which did not contain a methionine start codon, the reading frames of all sequenced genes encoded full-length proteins. In contrast to the other three molecular clones, which were obtained from biologically clonal infected peripheral blood lymphocyte cultures, clone 1 was obtained from infected H9 cells (13). A nonfunctional *vpu* gene is often found in molecular HIV-1 clones derived from cell lines (23). The maximum amino acid sequence variability for the separate HIV-1 genes is shown in Table 2. The *vif*, *vpr*, and *nef* genes showed only minor amino acid variation (1.0, 1.0, and 1.9%, respectively), confirming the genetic relationship of these clones (Table 2 and Fig. 1A, B, and F). In contrast, the *tat*, *rev*, and *vpu* genes showed a maximum amino acid variability of 7.9, 10.2, and 8.6%, respectively (Table 2). The amino acid variability in the *tat* gene was concentrated in the second exon (exon 1, 4.2%; exon 2, 17.2%) of this regulatory gene, which is located within the *env* gene. This amino acid variation, however, was not phenotype associated. In spite of the prominent amino acid variation in the *tat* and *rev* genes, the *tat* and *rev* proteins were completely homologous in regions thought to be critical for their functions (Fig. 1C and D) (9, 12, 14, 17, 22, 26). This is compatible with the finding that the clones did not exhibit significant differences in replication rate in peripheral blood lymphocytes (13). Although the sequence variability in the *vpu* gene was completely restricted to NSI clone 2.1, the absence of the initiator methionine codon of the *vpu* gene in SI clone 1 indicates that the *vpu* gene is not required for syncytium induction or for replication in T-cell lines and primary monocytes (Fig. 1E and Table 1).

Alignments of the deduced amino acid sequences of the *env* genes are shown in Fig. 2. Given the fact that all clones except clone 1 were derived from the same time point, the level of variation (up to 14.9% [Table 2]) was surprisingly high. Closer inspection, however, revealed the existence of four distinct domains within the *env* gene (termed A through D in Fig. 3). For each separate domain, the sequences of the eight clones fell apart into two distinct clusters (intercluster variation, 11 to 30%) with high internal similarities (96 to 100%). In each of the eight clones, the alleles of these four domains were combined in a unique manner, implying that exchange of genetic material had occurred. In particular, clones 1.1, 1.2, and 2.1 appeared to result from recombination events between a clone similar to clone 6 or 3 and a clone similar to clone 5 or 7 (Fig. 2 and 3). Clone 2.1 possessed striking sequence similarity to clones 6 and 3 between amino acids (aa) 87 and 426 (domain A in Fig. 3) and to clones 5 and 7 between aa 653 and 705 and aa 852 and 863 (domains C and D in Fig. 3). Clones 1.1 and 1.2 are highly similar to clones 5 and 7 between aa 87 and 426 (domain A in Fig. 3), to clones 6 and 3 between aa 599 and 738, and to clone 3 between aa 852 and 863 (Fig. 2 and 3). The transitions of sequence similarity between the HIV-1 variants were also observed at the nucleotide level (data not shown). Previously, it has been demonstrated that genetic recombination events between HIV-1 proviruses can occur in vitro (5). Since the *env* genes studied belonged to single-clone HIV-1 isolates recovered directly from patient peripheral blood mononuclear cells under limiting dilution conditions, these findings imply that recombination events occur in vivo as a mechanism to generate clonal diversity.

The *env* gene has been identified as the major determinant responsible for differences in biological properties (24, 28). To investigate whether in this set of molecular HIV-1 clones the *env* gene is involved in determining differences in the capacity to induce syncytia and cytotropism, chimeric proviruses were constructed. In the chimeric provirus 320.2A 1.2 *env* 2.1, an *NsiI-XhoI* fragment of clone 2.1 coding for amino acid residues 108 to 873 of the *env* gene and amino acid residues 1 to 43 of the *nef* gene was exchanged for the corresponding fragment of the Sup T1-tropic NSI clone 2.1. In the chimeric provirus 320.2A 1.2 *env* 1, the same fragment was replaced by the corresponding fragment of the H9- and Sup T1-tropic SI clone 1. Construction of the chimeric proviruses and determination of the biological properties of these constructs were performed as described elsewhere (13, 28). The finding that these two chimeric proviruses, with variations only within the *env* coding sequence, were different in their SI capacities and tropisms for the MT-2 and H9 T-cell lines provided proof that these phenotypic traits are conferred by determinants in the *env* gene (Table 3).

Comparison of the biological phenotypes of the eight clones and their differences in *env* gene sequence composi-

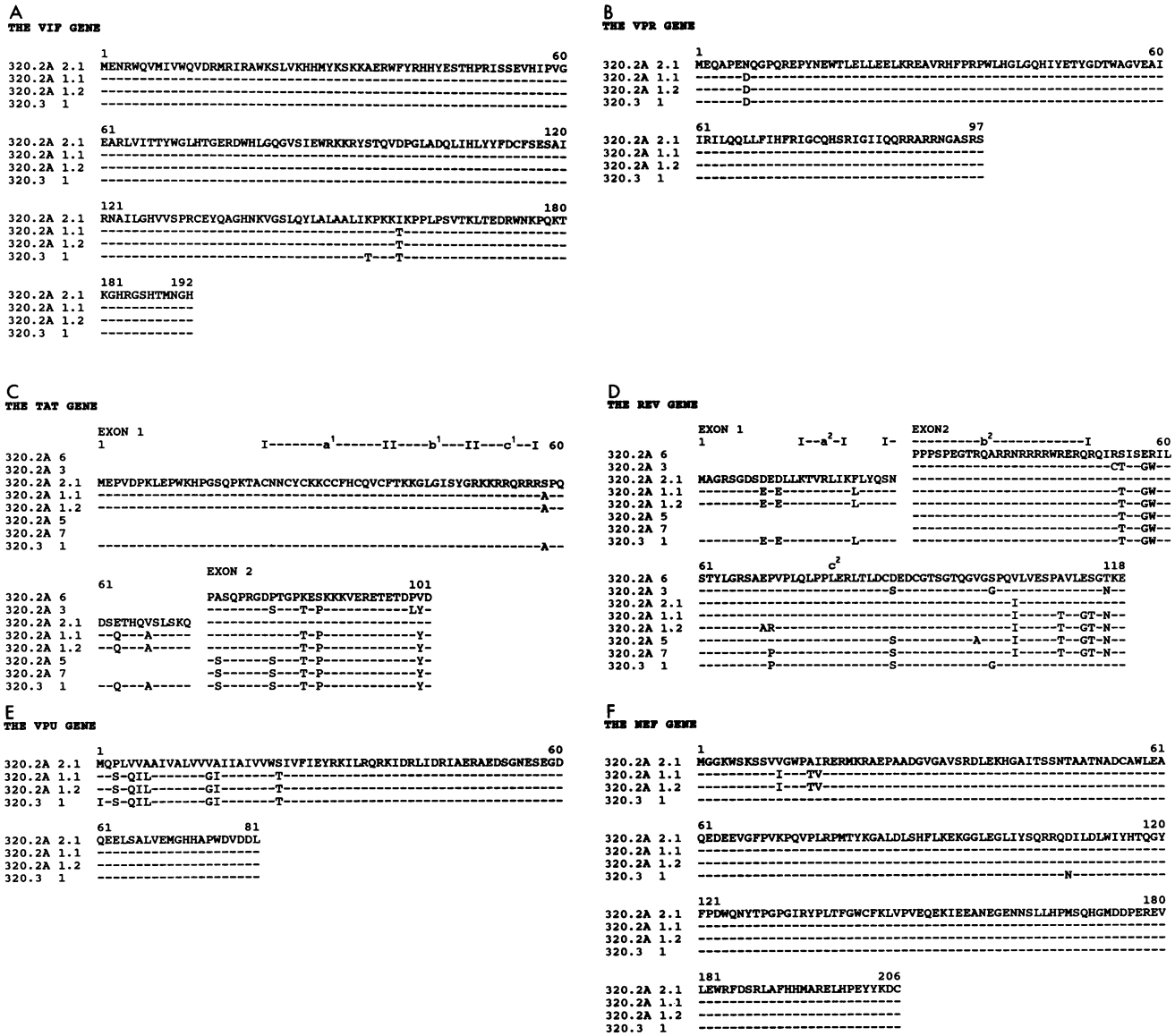

FIG. 1. Alignment of the predicted amino acid sequences of the *vif* (A), *vpr* (B), *tat* (C), *rev* (D), *vpu* (E), and *nef* (F) proteins of the molecular HIV-1 clones 2.1, 1.1, 1.2, and 1. For the biological clonal HIV-1 variants 6, 3, 5, and 7, the amino acid sequences of the second exons of the *tat* (C) and *rev* (D) genes are shown. Sequence analysis was performed by the dideoxy chain termination method using Sequenase (USB). Amino acid alignments were performed by using CLUSTAL (16). The predicted amino acid sequences are presented in the one-letter amino acid code. Amino acid numbers are indicated. Dashes indicate amino acid identities, whereas points indicate gaps. Functional regions of the *tat* and *rev* proteins are indicated. a^1, cysteine-rich region and proposed metal-binding region, which may mediate protein-protein interaction; b^1, possible activation domain; c^1, nucleolar localization signal and putative nucleic acid-binding region; a^2, region involved in nuclear localization and transactivation; b^2, nuclear localization signal possibly involved in binding to the *rev* responsive element; c^2, region near amino acid residues 78 and 79 involved in transactivation.

tion revealed a remarkable association between particular phenotypic traits and each of the four domains described above. On the basis of their sequences in the gp120 C1-V4 region (domain A in Fig. 3), the clones clustered into two groups, each with >98.5% internal homology, with one containing all SI MT-2-tropic clones and the other comprising all NSI non-MT-2-tropic clones. On the other hand, all H9-tropic clones clustered in the same group for domains A, C, and D, in contrast to all non-H9-tropic clones. In addition, clone 1, replicating most efficiently in H9 cells, had a unique sequence between 426 and 535 (domain B). The Sup T1-tropic clones 2.1, 5, 7, and 1 clustered in domains C and D. Clone 6, which replicated inefficiently in Sup T1 cells, clustered only in domain D with clones 2.1, 5, 7, and 1. Finally, clones that could not be transmitted to H9 or Sup T1 cells (3, 1.1, and 1.2) clustered in domains B, C, and D (100% similarity in all three regions), regardless of their abilities to induce syncytia or their MT-2 tropisms (Fig. 3). This indicates that the C1-V4 region of the gp120 molecule is responsible for differences in the capacity to induce syncytia and tropism for the MT-2 T-cell line, whereas determinants of tropism for the H9 and Sup T1 T-cell lines are located

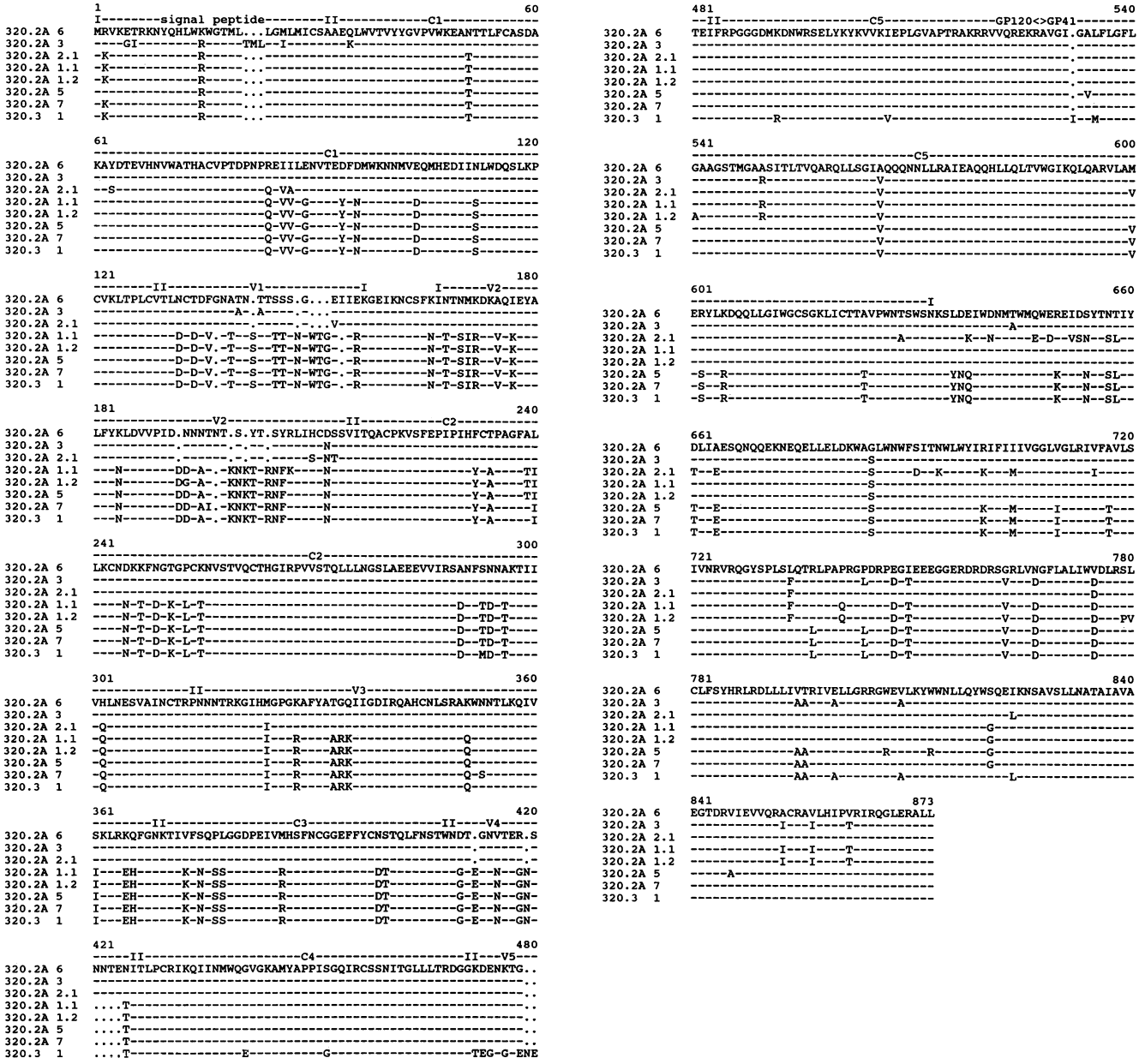

FIG. 2. Alignment of the *env* proteins of molecular and biological clonal HIV-1 variants 6, 3, 2.1, 1.1, 1.2, 5, 7, and 1. Sequence analysis was performed by the dideoxy chain termination method using Sequenase (USB). Amino acid alignments were performed by using CLUSTAL (16). Final alignments were adjusted by the eye. The predicted amino acid sequences are presented in the one-letter amino acid code. Amino acid numbers are indicated. Dashes indicate amino acid identities, whereas points indicate gaps. The signal peptide, hypervariable regions (V), constant regions (C), and gp120-gp41 proteolytic cleavage site are indicated.

downstream of aa 426 in the C-terminal portions of the gp120 and gp41 molecules. Although there is an association between particular phenotypic traits and the overall patterns of amino acid sequence arrangements in the four domains described, it remains possible that single amino acid alterations are responsible for the observed phenotypes. However, this possibility is heard to reconcile with the observed stability of recessive phenotypic traits of these clones in vitro.

Previous studies have identified regions of the gp120 glycoprotein involved in CD4 binding and association with the gp41 transmembrane glycoprotein. Amino acid residues Thr-272 and Trp-439 in hydrophobic domains of the C2 and C4 regions and amino acid residues Asp-381, Glu-383, and Asp-469 in hydrophilic domains of the C3 and C4 regions appear to be important for CD4 binding (25). Amino acid residues Val-38, Tyr-42, Trp-47, Ile-507, and Lys/Ala-516/517 located at the amino and carboxyl termini of the gp120 molecule contribute to the noncovalent association with the gp41 molecule (15, 19). The corresponding residues as well as the residues directly adjacent were completely conserved among the eight clones, except in clone 1, which possessed

TABLE 2. Maximum amino acid variability present in accessory and *env* genes of phenotypically divergent HIV-1 variants

Gene	Variability[a] (%)
vif	1.0
vpr	1.0
tat	7.9
rev	10.2
vpu	8.6
env	14.9
nef	1.9

[a] Variability was determined for the accessory genes of the four molecular HIV-1 clones and the *env* genes of the four molecular HIV-1 clones and the four biological HIV-1 clones.

TABLE 3. Biological properties of wild-type and chimeric HIV-1 proviruses

Molecular clone	SI in PBL[a]	Transmission to[b]:		
		MT-2	Sup T1	H9
2.1	−	−	+	−
1.2	(+)	+	−	−
1	+	+	+	+
1.2 *env* 2.1[c]	−	−	+	−
1.2 *env* 1[c]	+	+	+	+

[a] PBL, peripheral blood lymphocyte; −, no syncytia observed; (+), reduced SI capacity; +, SI.
[b] −, not transmissible; +, transmissible.
[c] aa 106 to 873 of the HIV-1 *env* gene and the first 34 aa residues of the HIV-1 *nef* gene were exchanged.

a Val instead of an Ile at position 507. Moreover, all clones exhibited an Arg instead of a Lys at position 516 (Fig. 2).

The amino terminus of gp41, which resembles the fusion peptides of ortho- and paramyxoviruses (11), has been proposed as a determinant of SI capacity (2, 21). However, the sequence identity between NSI clones 6 and 2.1 and SI clones 1.1 and 7 excludes the possibility that this region is the sole determinant of syncytium formation.

Recent studies indicate that the V3 loop not only is involved in type-specific neutralization but also might be a determinant of biological properties such as cytopathic properties and cellular tropism. Amino acid changes in the tip of the V3 loop affect the capacity to induce syncytia and alter cellular tropism (6, 10, 29). A high degree of sequence variation within the V3 loop was observed between the groups of NSI and SI clones. Recently, we have shown that SI HIV-1 variants contain V3 sequences with significantly higher positive charges than those of NSI HIV-1 variants (8). In the highly variable V3 domain, two amino acid residues located on either side of the V3 loop are responsible for these differences in charge. In NSI HIV-1 variants, these two residues are negatively charged or uncharged, whereas in SI HIV-1 variants, one or both are positively charged. This correlation is also observed in the presently studied NSI and SI HIV-1 variants (Fig. 2, aa 321 and 335). Experiments with chimeric proviruses suggested that the V3 loop is also a determinant for tropism for T-cell lines and primary monocytes (18, 24, 28, 32). However, the SI HIV-1 variants analyzed in the present study, which differ from each other in their tropisms for the H9 and Sup T1 T-cell lines and primary monocytes, possessed completely identical V3 regions (Fig. 2).

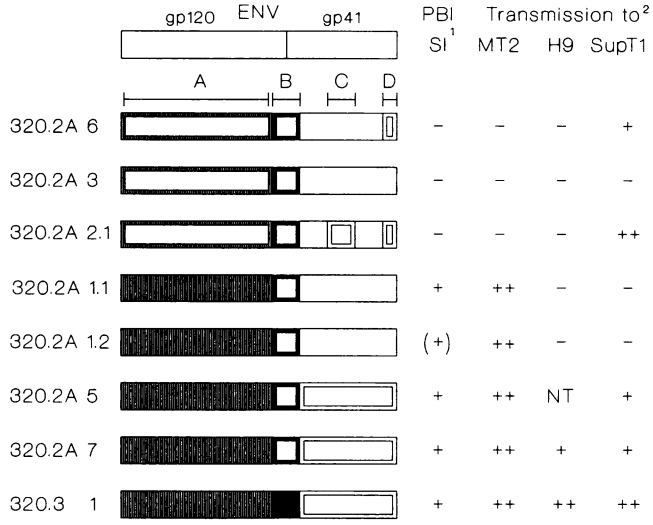

FIG. 3. Amino acid sequence similarity in *env* gene glycoproteins of the eight phenotypically heterogeneous HIV-1 variants 6, 3, 2.1, 1.1, 1.2, 5, 7, and 1. Sequence similarities are indicated by identical patterns. The minimum sequence similarities in regions with identical patterns are 98.5% for domain A (aa 87 to 426), 100% for domain B (aa 426 to 535), 96% for domain C (aa 653 to 705), and 100% for domain D (aa 852 to 863). Biological phenotypes of the corresponding molecular and biological clonal HIV-1 variants are indicated. [1]−, no syncytia observed; (+), reduced SI capacity; +, SI. [2]−, not transmissible; +, delayed growth kinetics; ++, readily transmissible; NT, not tested.

Analysis of these eight HIV-1 clones demonstrated extensive phenotype-associated sequence variation dispersed nonrandomly over the *env* gene. The observed considerable genetic distances between clones with different phenotypes may explain the stability of viral phenotypes even after prolonged propagation in vitro. Four clones with intermediate phenotypes most likely were the products of genetic recombination events in vivo, indicating that this mechanism, next to mutations resulting from errors during reverse transcription, may account for the emergence of proviruses with novel phenotypic properties during the course of the HIV-1 infection (31).

In agreement with previous reports (24, 28), the *env* gene appeared to be the major determinant responsible for differences in biological properties. The amino acids previously shown to be involved in gp120-CD4 binding and gp120-gp41 association and their directly surrounding residues were completely conserved among these eight clones. This argues against variation in gp120-CD4 binding affinity or variation in gp120 shedding as a cause of differences in the capacity to induce syncytia or cytotropism.

Sequence variation associated with SI capacity and tropism for the MT-2 T-cell line was concentrated in the C1-V4 region of the gp120 glycoprotein. In contrast, sequence variation associated with tropism for the H9 and Sup T1 T-cell lines was completely limited to the gp120-V5 region and the gp41 glycoprotein. In contrast to observations with other HIV-1 clones, the V3 region of the gp120 molecule of these clones appeared not to be critical for their tropisms for primary monocytes and the H9 and Sup T1 T-cell lines. This finding demonstrates the existence of additional determinants for cytotropism next to the V3 loop, most probably located within the gp120-V5 region and the gp41 molecule.

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